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Cyclooxygenase metabolites mediate glomerular monocyte chemoattractant protein-1 formation and monocyte recruitment in experimental glomerulonephritis[1]

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Cyclooxygenase metabolites mediate glomerular monocyte chemoattractant protein-1 formation and monocyte recruitment in experimental glomerulonephritis.

Background. Monocyte chemoattractant protein-1 (MCP-1) has been shown to play a significant role in the recruitment of monocytes/macrophages in experimental glomerulonephritis. Whereas a number of inflammatory mediators have been characterized that are involved in the expression of MCP-1 in renal disease, little is known about repressors of chemokine formation *in vivo*. We hypothesized that cyclooxygenase (COX) products influence the formation of MCP-1 and affect inflammatory cell recruitment in glomerulonephritis.

Methods. The effect of COX inhibitors was evaluated in the antithymocyte antibody model and an anti-glomerular basement membrane model of glomerulonephritis. Rats were treated with the COX-1/COX-2 inhibitor indomethacin and the selective COX-2 inhibitors meloxicam and SC 58125. Animals were studied at 1 hour, 24 hours, and 5 days after induction of the disease.

Results. Indomethacin, to a lesser degree the selective COX-2 inhibitors, enhanced glomerular MCP-1 and RANTES mRNA levels. Indomethacin enhanced glomerular monocyte chemoattractant activity an the infiltration of monocytes/macrophages at 24 hours and 5 days.

Conclusions. Our studies demontrate that COX products may serve as endogenous repressors of MCP-1 formation in experimental glomerulonephritis. The data suggest that COX-1 and COX-2 products mediate these effects differently because the selective COX-2 inhibitors had less influence on chemokine expression.

The infiltration and accumulation of leukocytes in glomeruli and the tubulointerstitium are typical pathological features of most immune-mediated glomerular injuries [1]. Recent experimental studies suggest that a group of cytokines called chemokines exhibit strong chemotactic activities and is involved in inflammatory cell recruitment into injured tissues [2, 3]. Among the chemokines, members of the -C-C- group, such as monocyte chemoattractant protein-1 (MCP-1), are strong attractants for monocytes [4]. Expression of MCP-1 has been demonstrated in glomeruli of animals and humans with glomerulonephritis and is involved in the monocyte/macrophage (M/M) infiltration into glomeruli and the renal interstitium [5–8]. The regulation of MCP-1 formation in glomerulonephritis, however, and the resulting pathophysiological consequences are unclear.

Recent *in vitro* studies of cultured mesangial cells have shown that several mediators of inflammation such as cytokines and growth factors can stimulate the release of MCP-1 [9–11]. In contrast, only a few repressors of MCP-1 expression have been described [12, 13]. In a rat model of antithymocyte antiserum-induced glomerulonephritis, the glomerular MCP-1 expression is reduced by prostaglandin E (PGE) [14]. These studies suggest that endogenously formed prostaglandins can modulate the formation of MCP-1 and influence the clinical outcome of the disease.

The major enzyme regulating the synthesis of prostaglandins is cyclooxygenase (COX) [15]. Two isozymes of COX, COX-1 and COX-2, have been characterized. COX-1 is a constitutive enzyme present in many mammalian cells [15, 16]. It is assumed that its products are important for the maintenance of normal functions in the kidney. COX-2 is undetectable in most mammalian tissues, but its expression can be rapidly and transiently induced by cytokines and growth factors in different cell types [17–19], including glomerular mesangial cells [20, 21]. These data suggest that COX-2 plays a major role in

[1] **See Editorial by Breyer, p. 738.**

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the rapid production of prostaglandins during inflammatory processes.

The assumption that COX products might be involved in the regulation of MCP-1 production must therefore include the consideration of products from both enzyme isoforms.

To investigate the possible role of COX-1 and COX-2 products in the glomerular formation of MCP-1 and the pathophysiological consequences, the effects of an unspecific COX-1/COX-2 inhibitor (indomethacin) and two selective COX-2 inhibitors [meloxicam (Mel) and SC 58125] were studied in two different models of glomerulonephritis in rats. A rat model of mesangial proliferative glomerulonephritis was studied at different time points after induction of the disease. The data were then confirmed in a model displaying a similar time frame of MCP-1 expression, a rat model of anti-glomerular basement membrane (GBM) nephritis.

Additionally, another -C-C- chemokine that may be involved in the recruitment of macrophages in glomerulonephritis, RANTES, was investigated [22].

The data reveal that the nonselective COX-inhibitor indomethacin stimulated the glomerular expression of MCP-1 and RANTES mRNA. This was paralleled by sustained glomerular recruitment of M/M. The selective inhibitors of COX-2 showed little effect on chemotactic activity and cell infiltration.

These findings suggest that the expression of glomerular MCP-1 might be repressed by COX products. The sustained glomerular MCP-1 formation with indomethacin, which is associated with enhanced chemotactic activity and an increased recruitment of M/M into glomeruli, underlines the important role of this chemokine in M/M infiltration in glomerulonephritis and demonstrates an important contribution of COX products as endogenous repressors of this proinflammatory cytokine.

METHODS

Compounds

Meloxicam and SC 58125 were gifts from Dr. Karl Thomae, Boehringer Ingelheim GmbH, Germany. Mel [23] and SC 58125 [24] are newly developed compounds that preferentially inhibit the COX-2 isoform. For that reason, they are called selective COX-2 inhibitors. Indomethacin, known to inhibit both COX isoforms with a slight selectivity for COX-1 [25], was purchased from Sigma Chemicals (Deisenhofen, München, Germany).

Induction of glomerulonephritis

Antirat thymocyte serum (ATS) nephritis. Immune-mediated mesangial cell injury was induced in male Wistar rats (120 to 150 g body wt; Charles River, Sulzfeld, Germany) by i.v. injection of 0.5 ml per 100 g body wt of a rabbit ATS. ATS was induced in New Zealand rabbits (Charles River) by repeated immunization with thymocytes from Lewis rats (Charles River). The specificity of the antibodies was tested *in vitro* and *in vivo* for their reactivity with mesangial cells. Details of the immunization as well as the characterization of the antiserum were described earlier [26]. The glomerulonephritis that develops after the intravenous administration of the antibody is characterized by an immune complex formation on mesangial cells, complement activation, and an infiltration of polymorphonuclear granulocytes and monocytes, resulting in mesangiolysis that is followed by mesangial cell proliferation and an increase in extracellular matrix. The morphologic and functional characteristics of this glomerular lesion have been described earlier [26].

Anti-GBM nephritis. Nephritis was induced in male Wistar rats (120 to 150 g body wt) by i.v. injection of 0.5 ml per 100 g body wt of a rabbit antirat glomeruli serum. The anti-GBM serum was induced in New Zealand rabbits by repeated immunization (four times) with a rat GBM preparation. For each immunization, glomeruli derived from two rat kidneys (1.0 g each) were isolated using a differential sieving technique as described earlier [27]. Glomeruli were washed twice in cold phosphate-buffered saline (PBS) and were collected in 1 ml of PBS. Then glomeruli were homogenized in a Wheaton dounce tissue homogenizer (Wheaton, Millville, NJ, USA) using a loose fit pestel. Glomerular homogenate was sonified twice for 10 seconds (Branson Sonifier 250; Branson, Danbury, CT, USA) at 20% energy output level. After sonification, the preparation was subjected to a second dounce homogenization using a tight fit pestle. One milliliter of glomerular homogenate containing rat GBM fragments was mixed with 0.5 ml of immunizing adjuvants (Hunter's Titer Max; Boehringer Ingelheim Bioproducts, Heidelberg, Germany), and rabbits were injected with this preparation. One week after the last immunization, rabbits were bled. The antiserum was heat inactivated and was kept frozen at −20°C in aliquots. Antigen specificity was evaluated for each antibody preparation *in vivo* for their reactivity with GBM (data not shown). The glomerulonephritis that develops after the intravenous administration of the antibody is characterized by a linear deposition of antibodies at the GBM, complement activation, and an infiltration of polymorphonuclear granulocytes and monocytes.

Animal groups and experimental protocol

ATS nephritis. Five groups of animals were studied at each time point. Four animals were studied per group at each time point. All time points were investigated at least twice. Experiments in anti-GBM nephritis were carried out only at five days after induction of the disease with four groups of animals, consisting of five animals in each group.

Control rats received 0.5 ml per 100 g body wt of nonantibody IgG intravenously.

All nephritic rats received 0.5 ml per 100 g body wt of antithymocyte serum or 0.4 ml per 100 g body wt of anti-GBM antiserum, respectively, intravenously. Control and nephritic rats received 0.5 ml of 1% methylcellulose orally twice daily.

Nephritic rats treated with Mel received 2 mg/kg body wt of Mel in 0.5 ml 1% methylcellulose orally twice daily. Nephritic rats treated with SC 58125 (only in ATS nephritis) received 2 mg/kg body wt of SC 58125 in 0.5 ml 1% methylcellulose orally twice daily. This dose was chosen because of its reported anti-inflammatory effect [24]. In another set of experiments in ATS nephritis, rats received either 3 mg/kg or 6 mg/kg body wt of SC 58125 in 0.5 ml 1% methylcellulose orally twice daily. Nephritic rats treated with indomethacin received 6 mg/kg body wt (2 mg/kg body wt in the group treated for five days) of indomethacin in 0.5 ml of a 5% buffered 0.5 M Na_2HPO_4/NaH_2PO_4 solution orally twice daily. In all groups, oral medication was started 12 hours before induction of the disease and continued until sacrifice.

One hour, 24 hours, and 5 days following the application of the antibody, animals were sacrificed for collection of renal tissue. Kidneys were removed after perfusion *in situ* with 50 ml 1 × PBS. Glomeruli were isolated from each kidney by a fractional sieving technique as described elsewhere [27]. Glomeruli were then separated for extraction of total RNA, Western blotting, and incubation with cell culture medium. At 1 hour, 24 hours, and 5 days, tissue from one kidney of each rat was collected for histologic evaluation. Urine excretion over 12 hours was collected in metabolic cages. The urines were centrifuged at 3,500 r.p.m. for five minutes and were frozen at −20°C until determination of PGE_2.

Histology

In the ATS experiments, renal tissue was obtained from all animals at 1 hour, 24 hours, and 5 days following induction of the disease to assess binding of ATS and complement C3 and to evaluate glomerular M/M infiltration. In the anti-GBM experiment, renal tissue was obtained from all animals at 5 days following induction of the disease to assess deposition of antiserum and to evaluate glomerular M/M infiltration. The tissue was fixed in 4% buffered formaldehyde and in methyl Carnoy's solution, respectively. To control for ATS, anti-GBM antiserum, and rat C3 binding in kidneys, paraffin-embedded tissue sections (2 μm) were stained with a goat antibody directed against rabbit IgG (Dakopatts, Hamburg, Germany) and a mouse monoclonal antibody against rat C3 (Nordic, Tilborg, the Netherlands). To analyze the infiltration of M/M into nephritic glomeruli, kidney tissue was stained with an antibody directed against the monocyte-specific marker ED-1 (Chemicon International, Temecula, CA, USA). Tissue sections were developed with the alkaline phosphatase antialkaline phosphatase technique. The appearance of ED-1–positive cells was assessed by counting positive cells in at least 50 glomeruli per kidney by light microscopy in a blinded fashion. Eight different kidneys of each group were evaluated at 24 hours and 5 days in the ATS experiments, and five different kidneys of each group at five days in the anti-GBM experiment. The cell numbers of ED-1–positive cells are given per glomerular cross section as means ± SEM.

Isolation of total RNA and Northern blot hybridization

After isolation of glomeruli, cellular RNA from four to six pooled kidneys was prepared by the guanidinium isothiocyanate method [28]. Twenty-five micrograms of total RNA were electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde. Equal loading of lanes was evaluated by ethidium bromide staining of the 18S and 28S rRNA. The RNA was transferred to nylon membranes (Zetabind; Cuno, Meriden, CT, USA) by vacuum blotting and was UV cross-linked. The membranes were hybridized with a cDNA probe for a rat 0.42 kb MCP-1 fragment [29] or a cDNA probe for a murine 0.6 kb RANTES fragment [30] after [^{32}P]dCTP-labeling by random oligonucleotide priming of the cDNA insert in RapidHyb-buffer (Amersham, Braunschweig, Germany) for 18 hours at 72°C. The membranes were washed in 2 × SSC − 0.5% SDS (20 × SSC is 3 M sodium chloride − 0.3 M sodium citrate) for 20 minutes at room temperature, then in 0.4 × SSC − 0.5% SDS and subsequently in 0.1 SSC − 0.1% SDS at 72°C for 20 minutes each. Autoradiography was performed with intensifying screens at −70°C for 2 to 24 hours. The size of the respective RNA was identified by comparison of its mobility with the ethidium bromide-stained RNA standards. The membranes were stripped for 20 minutes in 5 mM Tris-HCl (pH 8.0), 0.5% sodium pyrophosphate, 5 × Denhardt's solution, and 0.2 mM ethylenediaminetetraacetic acid (pH 8.0) at 65°C and were rehybridized with a 0.58 kb Eco RI cDNA probe encoding for human 18S rRNA to account for small loading and transfer variations. Exposed films were scanned with a laser densitometer (Hoefer Scientific Instruments, San Francisco, CA, USA), and relative RNA levels were calculated. Results are given as the mean of the independent investigations.

Determination of PGE_2 in the supernatant of isolated glomeruli

After isolation, glomeruli of one-half kidney were incubated with 1.5 ml RPMI 1640 tissue culture medium at 37°C for 40 minutes. To determine if there was a possible inhibition of PGE_2 production by COX inhibi-

tors *in vitro*, either indomethacin (1 μM) or Mel (1 μM) were added to the medium of incubation. After centrifugation at 3,500 r.p.m. for 10 minutes, the supernatant was collected, and glomeruli and supernatant were frozen at −20°C until determination of protein and PGE_2. Protein was determined after solubilizing glomeruli in 0.5 N NaOH using a BioRad Protein assay. PGE_2 was determined using a PGE_2 EIA (Cayman Chemical Co., Ann Arbor, MI, USA). The results are expressed as pg PGE_2 per μg total cell protein per minutes.

Determination of urinary PGE_2

Prostaglandin E2 concentration in the urine from animals placed in metabolic cages overnight (12 hours) was determined using the PGE_2 EIA (Cayman Chemical Co.). The total amount of PGE_2 in the urine was calculated by multiplication of the measured concentration by the volume of the excreted urine. Results are expressed as pg PGE_2.

Monocyte chemotactic activity in conditioned media of isolated glomeruli

To study the release of chemotactic activity from glomeruli at 24 hours and 5 days after induction of the ATS glomerulonephritis and 5 days after induction of the anti-GBM nephritis, glomeruli from control rats, nephritic rats, and nephritic rats treated with Mel, SC 58125 (not in anti-GBM nephritis), and indomethacin, respectively, were isolated as described earlier here. Following isolation, glomeruli of approximately one kidney were incubated in serum-free Dulbecco's modified Eagle's medium for 4.5 hours at 37°C in 5% CO_2/95% air. Conditioned media and glomeruli were collected following centrifugation of the glomeruli and frozen at −20°C until evaluation for chemotactic activity. Glomeruli of each preparation were counted, and the protein content of collected glomeruli was determined with a modified Lowry method (BioRad protein assay) to relate chemotactic activity to glomeruli and protein content of each preparation.

Monocyte chemotactic activity was determined in modified Boyden chambers (Poretics, Livermore, CA, USA) using freshly prepared human peripheral mononuclear cells as described earlier [31]. Blood was drawn into acid-citrate-dextrose and was centrifuged at 550 g for 20 minutes. The cell pellet was resuspended in Hank's balanced salt solution (HBSS) containing 0.27% dextrose and 0.37% sodium citrate and layered over one volume of histopaque-1077 (Sigma) and was centrifuged at 400 g for 40 minutes. The mononuclear cell layer was washed in HBSS and was resuspended in RPMI-0.2% albumin to 3.5×10^6 cells/ml. The monocyte cell chemotaxis was quantitated on 8 μm pore polycarbonate filters in blind-well chambers (Poretics). Solutions of conditioned media from glomeruli were diluted with RPMI-0.2% albumin, and 200 μl were placed in the lower compartment of the chambers. Monocytes (500 μl, 1.75×10^6 cells) were added to the upper compartments above the filters. The chambers were incubated at 37°C for 90 minutes in a humidified 5% CO_2 atmosphere. The filters were removed and were stained with Giemsa, and chemotaxis was assayed by counting the number of cells on the attractant side of the filter in 10 oil-immersion fields. Chemotactic activity is expressed as the mean number of monocytes migrating per field and per mg glomerular protein.

Western blots of COX-1 and COX-2

For Western blotting, glomeruli were isolated and centrifuged in 1 × PBS. A modification of Western blotting [32] was used as follows. The pellet was resuspended in 150 μl of Laemmli buffer 1 (33% 0.5 mM Tris-HCl pH 6.8, 66% SDS 10%). Samples were boiled for 10 minutes and were centrifuged. The protein concentration was determined with a modified Lowry method (Protein DC-assay; Biorad). To equal amounts of protein (100 μg), 1/4 vol% Laemmli buffer 2 (50% β-mercaptoethanol, 50% glycerol) and 1/5 vol% staining solution (42.5% glycerol, 0.5% bromphenol blue) were added. The solution was electrophoresed on a 8% SDS/polyacrylamide gel. A low molecular weight marker (Rainbow marker; Amersham), which compromises 14.3 to 200 kDa, served as the molecular-weight standard. A mouse macrophage cell lysate served as a positive control for COX-2. After the completion of electrophoresis, proteins were electroblotted semidry (Anode buffer I: 30 mM Tris, 20% methanol; Anode buffer II: 300 mM Tris, 20% methanol; Cathode buffer: 25 mM Tris, 40 mM 6-aminohexane acid, 20% methanol) for 60 minutes at 0.8 mA/cm2 to a nitrocellulose membrane (Hybond ECL; Amersham). The membrane was blocked with 5% nonfat dry milk in washing buffer (1 × PBS, 0.1% Tween 20) for one hour at room temperature and then was incubated for another hour with a monoclonal IgG 1 mouse-antirat COX-2 antibody (Transduction Laboratories, Lexington, KY, USA) in a concentration of 1:250 or a polyclonal goat-antimouse COX-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) added to a concentration of 1:500 in the same buffer. After rinsing the membrane in washing buffer for 3 × 8 minutes, the secondary antibody, a rabbit antimouse-immunoglobulin G (Transduction Laboratories) or a rabbit antigoat-immunoglobulin (Southern Biotechnology, Birmingham, AL, USA) conjugated to horseradish-peroxidase was added at a concentration of 1:1000. The luminescence detection of peroxidase was performed with the ECL system according to the manufacturer's recommendations (Amersham) after rinsing membranes in washing buffer and 1 × PBS. Films were exposed 30 seconds to 10 minutes at room temperature. After final exposure, staining with Ponceau S confirmed equal transfer of proteins.

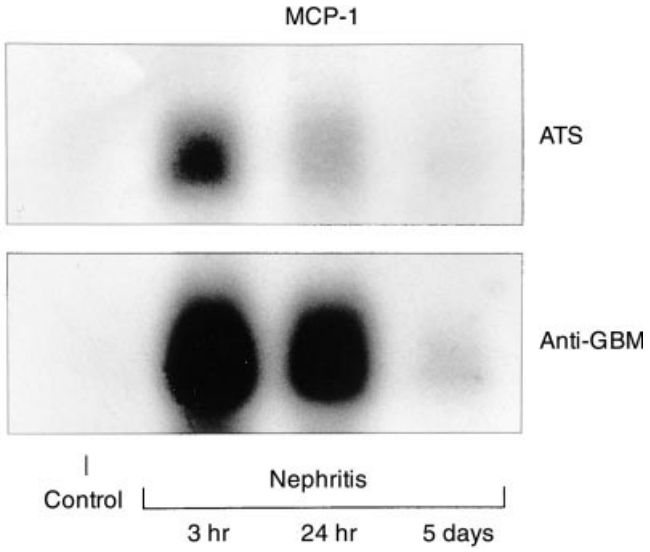

Fig. 1. Expression of mRNA of monocyte chemoattractant protein-1 (MCP-1) during the time course of the anti-thymocyte antibody (ATS) and anti-glomerular basemement membrane (anti-GBM) nephritis. MCP-1 mRNA levels increased significantly at 3 hours and 24 hours and returned to almost normal levels at 5 days. Equal loading transfer of each lane was confirmed by rehybridization against 18S rRNA (not shown).

Statistical analysis

Results are expressed as mean ± SEM unless stated otherwise. Statistical significance was defined as $P < 0.05$. We applied Wilcoxon-Mann-Whitney test to compare two distinct treatment groups (that is, nephritic vs. control or nephritic vs. treated nephritic animals).

RESULTS

Northern blotting

Monocyte chemoattractant protein-1 mRNA expression, evaluated by Northern blotting, increased significantly in nephritic animals at 3 hours and 24 hours after induction of the disease when compared with controls in both models of glomerulonephritis (ATS and anti-GBM). At 5 days MCP-1, mRNA levels returned almost to control levels (Fig. 1).

In the ATS nephritis, the pattern of MCP-1 expression changed markedly under the influence of the COX inhibitors at 24 hours and 5 days, whereas treatment with selective and nonselective inhibitors of COX did not significantly modify the expression of MCP-1 mRNA at one hour (Fig. 2). Indomethacin enhanced the MCP-1 expression at 24 hours (twofold; Fig. 2), an effect that was more pronounced at day 5 (fivefold; Fig. 2) when compared with untreated nephritic animals. The more selective COX-2 inhibitors Mel and SC at a dosage of 2 mg/kg body wt showed less systematic effects on glomerular MCP-1 mRNA levels at 24 hours and 5 days (Fig. 2). However, when used in higher doses, the selective

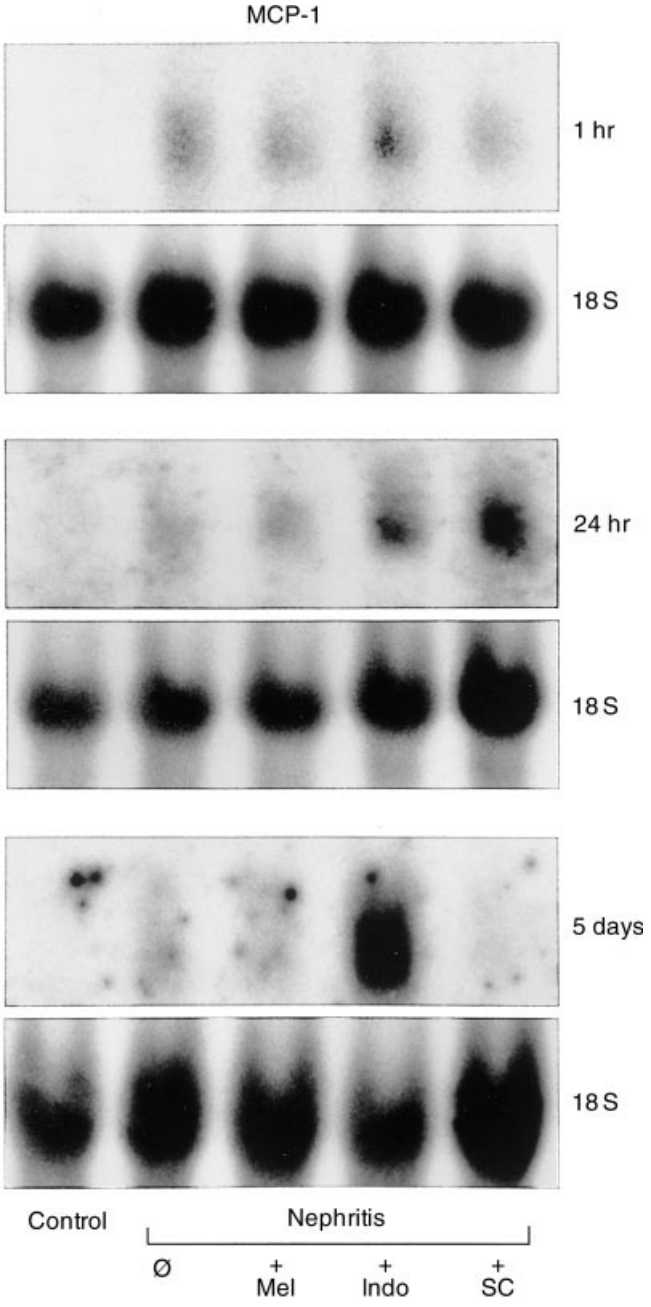

Fig. 2. Expression of mRNA of MCP-1 from isolated glomeruli of ATS nephritic rats 1 hour, 24 hours, and 5 days after induction of the disease under the influence of the treatment with the unselective COX-1/COX-2 inhibitor indomethacin (Indo) or the selective COX-2 inhibitors meloxicam (Mel) or SC 58125 (SC). MCP-1 mRNA levels are increased by treatment with indomethacin at 24 hours (twofold), and more significantly at 5 days (fivefold).

COX-2 inhibitor SC 58125 also enhanced glomerular MCP-1 mRNA levels at day 5 (Figs. 3 to 6), and the differences between the selective compound and unselective COX-1/COX-2 inhibitor indomethacin were diminished (SC 58125, twofold at 3 mg/kg and 2.5-fold at 6 mg/kg; indomethacin, 2.7-fold at 2 mg/kg when compared with untreated nephritis; Fig. 6).

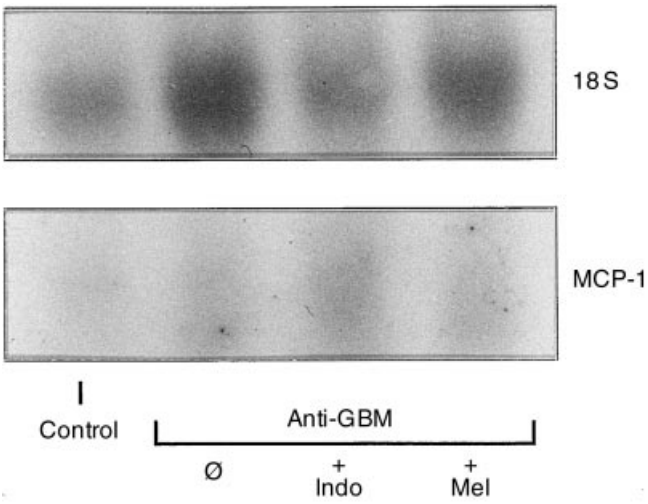

Fig. 3. Expression of MCP-1 mRNA from isolated glomeruli of rats 5 days after induction of the anti-GBM nephritis under the influence of treatment with the unselective COX-1/COX-2 inhibitor indomethacin (Indo) or the selective COX-2 inhibitor meloxicam (Mel). MCP-1 mRNA levels are increased in nephritic animals and are further enhanced by treatment with indomethacin (threefold in comparison to untreated nephritis when corrected for 18S rRNA).

Similarly, as found in the ATS model, MCP-1 mRNA expression is also enhanced by indomethacin in the anti-GBM nephritis at five days after induction of the disease when compared with untreated nephritis (threefold; Fig. 3).

RANTES mRNA expression was moderately increased in glomeruli from nephritic animals at 24 hours, and more so at 5 days after induction of the disease (data not shown). At 5 days, treatment with indomethacin significantly augmented RANTES mRNA expression (twofold when compared with untreated nephritis). The selective COX-2 inhibitor SC 58125 induced a smaller increase (1.4-fold at 3 mg/kg, 1.35-fold at 6 mg/kg body wt when compared with untreated nephritis) of glomerular RANTES expression (Fig. 6).

Monocyte chemotactic activity of glomerular supernatants

In the ATS model, the monocyte chemotactic activity of conditioned media from nephritic glomeruli was significantly higher when compared with controls at 24 hours (1.38 MØ/mg protein in nephritic glomeruli vs. 0.53 in controls), but returned to control levels at 5 days (Table 1). The stimulated chemotactic activity was further augmented by indomethacin at 24 hours (4.98 MØ/mg protein vs. 1.38 in controls) and at 5 days (2.34 MØ/mg protein vs. 0.63 in nephritic glomeruli). Treatment of the nephritic animals with the selective inhibitors Mel and SC did not affect chemotactic activity to the same degree as indomethacin at any time point (Table 1). Even when used at a higher dose (6 mg/kg body wt) in an additional experiment, SC 58125 showed less chemotactic activity than indomethacin (24 hr, 2.35 MØ/mg protein in SC 58125 vs. 5.1 in animals treated with indomethacin; 5 days, 1.74 MØ/mg protein in SC 58125 vs. 3.40 in animals treated with indomethacin) (see also Fig. 5).

In the anti-GBM model, the monocyte chemotactic activity of conditioned media from glomeruli of nephritic animals treated with indomethacin was higher when compared with untreated nephritic animals at 5 days (1.48 MØ/mg protein in indomethacin-treated glomeruli vs. 0.55 in untreated nephritic glomeruli; Table 1).

Western blot analysis for COX-1 and COX-2

The influence of unselective COX-1/COX-2 and selective COX-2 inhibition on glomerular COX-1 and COX-2 protein levels was evaluated in both the ATS and anti-GBM nephritis at 5 days following the induction of the disease. COX-1 protein levels were not modified in nephritic animals or nephritic animals treated with selective COX-2 inhibitors. Indomethacin markedly reduced glomerular COX-1 protein levels at 5 days in both models (Fig. 4A, ATS nephritis; Fig. 4B, anti-GBM nephritis). COX-2 protein levels were slightly increased by all inhibitors in both models (Fig. 4).

PGE_2 formation

Release of PGE_2 in the supernatant of isolated glomeruli from controls, ATS nephritic rats and ATS nephritic rats treated with indomethacin or Mel at 24 hours and 5 days, is shown in Table 2. Because none of the COX inhibitors studied bind covalently to the enzymes, release of PGE_2 in the supernatant reflects the enzyme activity of *in vivo* pretreated glomeruli in the absence of the inhibitors. At 24 hours following induction of the glomerulonephritis, PGE_2 release of glomeruli from nephritic animals was not significantly different compared with controls. At 5 days, however, the PGE_2 release from nephritic glomeruli was twice as much as from glomeruli of control animals. Glomeruli isolated at 24 hours from animals treated with Mel or indomethacin produced significantly more PGE_2 when compared with nephritic or untreated animals, an effect that was not seen in glomeruli isolated at 5 days. To ensure that COX inhibition with the drugs used was effective, Mel and indomethacin were added at a concentration of 1 μM to the isolated glomeruli of all animal groups studied. Both inhibitors significantly reduced prostaglandin formation below baseline control levels (data not shown). To evaluate the effectivity of the COX inhibitors *in vivo*, urinary PGE_2 excretion was determined at 5 days. At this time point, indomethacin significantly inhibited PGE_2 excretion, whereas the selective COX-2 inhibitors Mel and SC did not (Table 3).

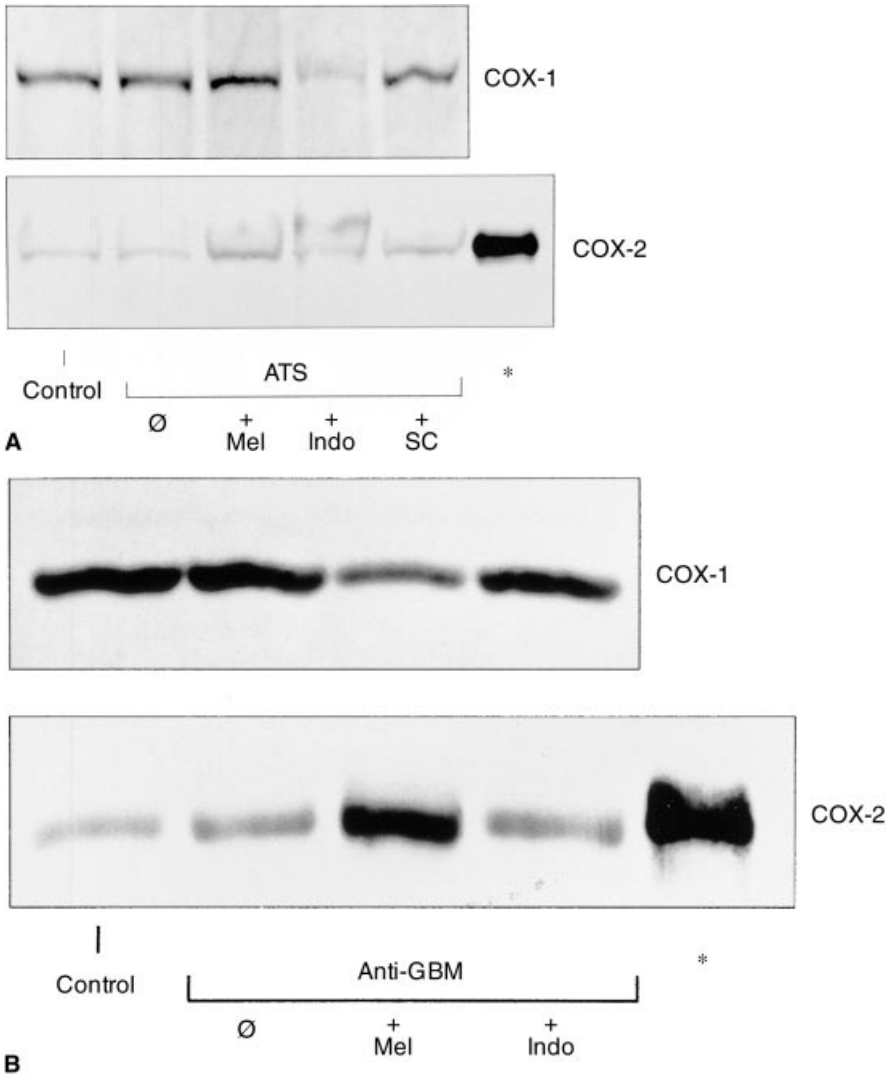

Fig. 4. Expression of COX-1 and COX-2 protein levels at five days after induction of the disease under the influence of the treatment with the unselective COX-1/COX-2 inhibitor indomethacin (Indo) or the selective COX-2 inhibitors meloxicam (Mel) or SC 58125 (SC). Treatment with indomethacin decreases COX-1 protein levels, whereas COX-2 protein levels are slightly increased. Treatment with SC 58125 or meloxicam does not decrease COX-1 levels. (*A*) ATS nephritis. (*B*) Anti-GBM nephritis. A mouse macrophage cell lysate (*) served as positive control for COX-2 protein.

Histology

Induction of ATS nephritis was followed by glomerular infiltration of ED-1–positive cells, with a maximum at 24 hours (Table 4). Treatment of ATS nephritic rats with selective inhibitors of COX-2 did not significantly modify the number of infiltrating ED1(+) cells at 24 hours or 5 days. In contrast, treatment of ATS nephritic rats with indomethacin increased the recruitment of ED1(+) cells significantly both at 24 hours and 5 days (Table 4).

Additional experiments in the anti-GBM nephritis also show that treatment with indomethacin significantly increased the number of infiltrating ED1 (+) cells at 5 days after induction of the nephritis (Table 4).

DISCUSSION

Monocyte chemoattractant protein-1 is a chemokine that plays an important role in monocyte chemoattraction in glomerulonephritis [5–8, 33]. In the antithymocyte antibody-induced model of glomerulonephritis, the expression of MCP-1 is positively correlated with the infiltration of M/M in glomeruli, and the disappearance of MCP-1 expression is followed by a reduction of glomerular monocytes [8]. This suggests a regulation of the disappearance of MCP-1 in this disease, either by a reduction of MCP-1–stimulating factors or by an enhanced or novel appearance of repressive factors of MCP-1 formation, thus contributing to the healing process in this disease. A better understanding of the regulation of stimulating or repressing factors might be important for the development of therapeutic strategies. In contrast to the large number of proinflammatory mediators that have been suggested as being responsible for the stimulation of MCP-1 in glomerulonephritis [9–11], so far no information is available on potential repressors of MCP-1 that might be operative even in the situation of persistently

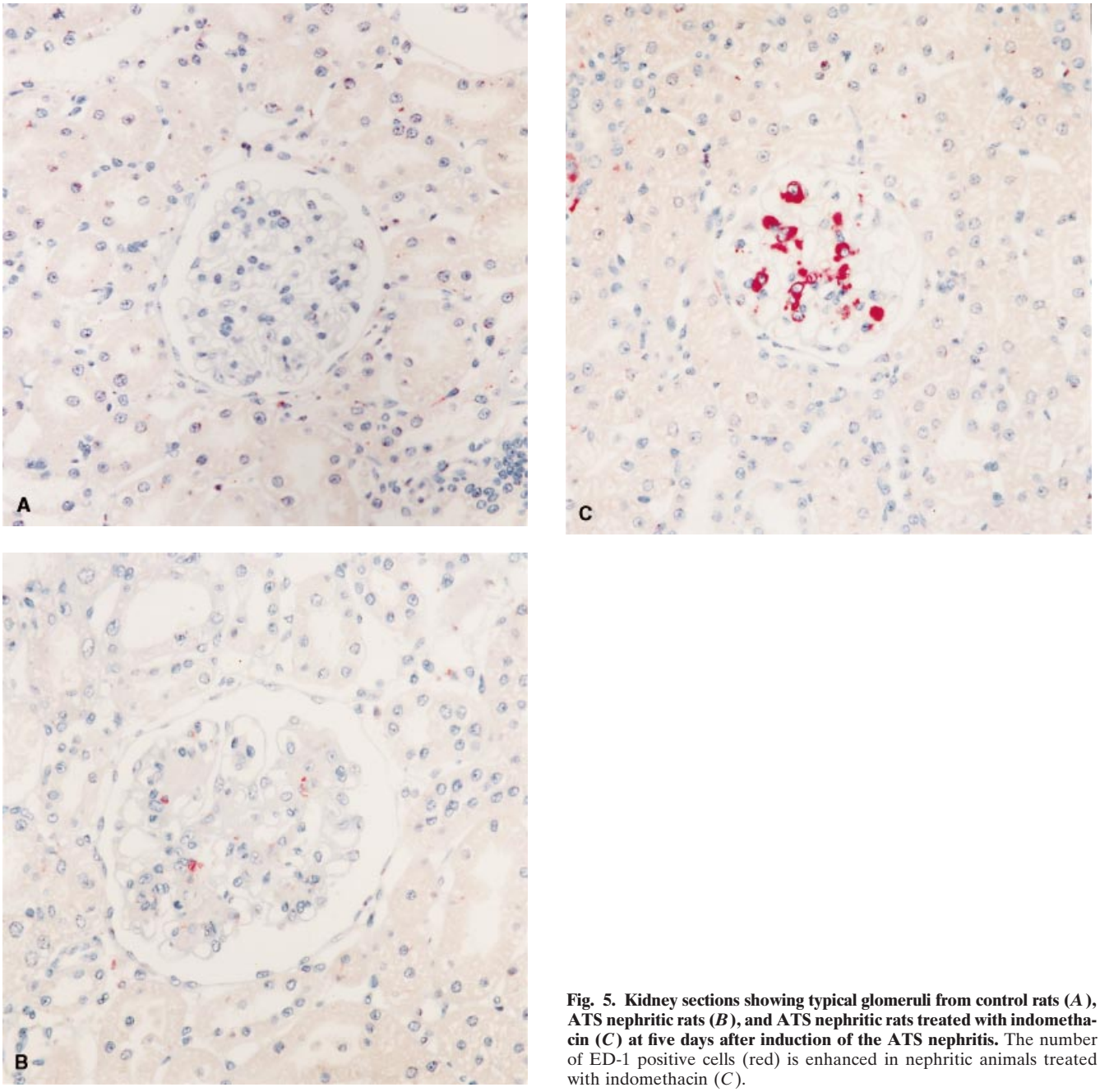

Fig. 5. Kidney sections showing typical glomeruli from control rats (*A*), ATS nephritic rats (*B*), and ATS nephritic rats treated with indomethacin (*C*) at five days after induction of the ATS nephritis. The number of ED-1 positive cells (red) is enhanced in nephritic animals treated with indomethacin (*C*).

increased proinflammatory mediators. Such hypothetical endogenous repressors might be responsible for a down-regulation of MCP-1 expression with the consecutive effect of diminished M/M recruitment. Recent evidence in cell culture studies [11] and *in vivo* experiments in the anti-thy-1 model [14] indicate that PGs of the E series suppress the formation of MCP-1 and reduce the infiltration of inflammatory cells into glomeruli. Because prostaglandins are major local products in the kidney [34], they might serve as endogenous repressors of MCP-1. To test this hypothesis, rats with the ATS-induced glomerulonephritis were treated with COX inhibitors, and the formation of MCP-1 was evaluated in glomeruli in conjunction with histologic studies. To study whether possible changes have a more general role in glomerulonephritis, we added experiments in another model displaying a similar pattern of MCP-1 expression (Fig. 1), the anti-GBM nephritis in rats. To control for a possible similar regulation of other chemokines involved in the recruitment of inflammatory cells, expression of RANTES was evaluated under the influence of COX inhibition in the ATS model. As for MCP-1, there was a similar rise

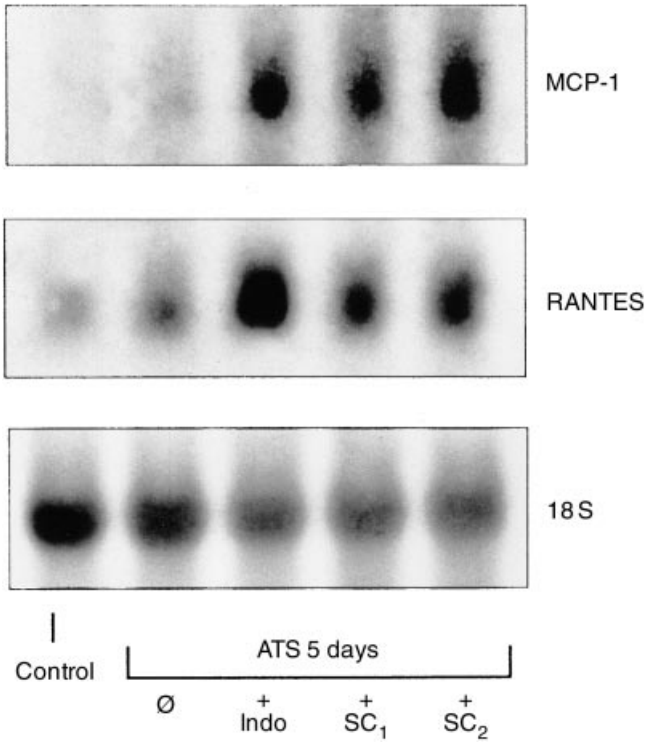

Fig. 6. Expression of MCP-1 and RANTES mRNA from isolated glomeruli of rats five days after induction of the ATS nephritis under the influence of treatment with the unselective COX-1/COX-2 inhibitor indomethacin (Indo, 2 mg/kg body wt) or the selective COX-2 inhibitor SC 58125 at higher doses (SC_1 3 mg/kg, SC_2 6 mg/kg body wt). Indomethacin increases MCP-1 mRNA levels (threefold) and RANTES mRNA levels (twofold) in comparison to untreated nephritic animals. SC 58125 shows a dose-dependent effect, increasing MCP-1 levels twofold at 3 mg/kg and 2.5-fold at 6 mg/kg, an effect that is less pronounced for RANTES mRNA levels (SC_1 1.4-fold, SC_2 1.35-fold) when compared with untreated nephritic animals.

Table 1. Monocyte chemotactic activity

	Time	Monocytes	SD
Control		0.53	0.26
ATS	24 hours	1.38	0.13
ATS + Mel	24 hours	2.20	1.51
ATS + Indo	24 hours	4.98	2.38[a]
ATS	5 days	0.63	0.20
ATS + Mel	5 days	0.54	0.08
ATS + Indo	5 days	2.34	1.30[b]
ATS + SC	5 days	0.69	0.15
Control		0.47	
Anti-GBM	5 days	0.55	
Anti-GBM + Mel	5 days	0.91	
Anti-GBM + Indo	5 days	1.48	

Monocyte chemotactic activity of conditioned media from glomeruli of control, nephritic, and nephritic animals treated with COX-inhibitors [indomethacin (Indo), meloxicam (Mel), SC 58125 (SC)] isolated at 24 hours and 5 days after induction of the antirat thymocyte serum (ATS) nephritis and at 5 days of the anti-GBM nephritis. ATS animals revealed an increase of chemotactic activity at 24 hours but returned to control levels at 5 days. Indomethacin further enhanced chemotactic activity at 24 hours and 5 days, whereas SC 58125 and meloxicam had no effect. Data are given as the mean number of migrated monocytes/high power field/mg glomerular protein from 3 different experiments. Additional experiments in the anti-GBM nephritis also reveal an enhanced glomerular-derived chemotactic activity by treatment with indomethacin at 5 days.

[a] $P = 0.01$ vs. ATS

[b] $P = 0.05$ vs. ATS and vs. ATS + Mel or + SC

Table 2. Prostaglandin E_2 (PGE_2) release from isolated glomeruli

	Time	PGE_2 *pg/min/mg protein*	SD
Control		11.63	5.90
Nephritis	24 hours	8.87	3.30
Nephritis + Mel	24 hours	27.70	13.80[a]
Nephritis + Indo	24 hours	22.67	8.40[a]
Control		10.38	2.61
Nephritis	day 5	21.24	13.60
Nephritis + Mel	day 5	29.91	13.60
Nephritis + Indo	day 5	34.77	11.73[b]
Nephritis + SC	day 5	16.14	6.27

PGE_2 release in the supernatants of isolated glomeruli from control rats, nephritic rats, and nephritic rats treated with indomethacin (Indo) or meloxicam (Mel). At 24 hours there was less glomerular PGE_2 production in nephritic rats than in controls, at 5 days nephritic glomeruli produced twice as much PGE_2 as controls; however, these effects did not reach statistical significance. Animals treated with either Indo or Mel released more prostaglandins than nephritic rats at 24 hours, an effect that was not significant at 5 days. Results are expressed as pg PGE_2/min of incubation time/mg of glomerular protein. PGE_2 release in the supernatants was significantly inhibited in all groups to levels below 5.24 pg/min/mg protein when indomethacin or meloxicam were added *in vitro* to the medium of incubation at a concentration of 1 μM (data not shown).

[a] $P < 0.001$ vs. nephritis

[b] $P < 0.03$ vs. nephritis + SC

in mRNA expression of RANTES under the influence of COX inhibition. These data suggest that COX products do effect the expression of more than one chemokine.

The major findings of our study are that the unselective COX-1/COX-2 inhibitor indomethacin increases the MCP-1 mRNA expression, enhances the release of chemoattractant activity, and augments the M/M infiltration in glomeruli of nephritic animals. We suggest that indomethacin exerts these effects by reducing the *in vivo* production of renal prostaglandins and that this can be attributed to the blockade of COX products. This suggestion is supported by the observation that PGE_2 production, as the main COX product of the glomerulus [34], is maximal at day 5 (Table 2) where almost no MCP-1 expression is detectable, whereas MCP-1 and chemotactic activity is maximal (Table 1) when PGE_2 is almost unchanged or reduced (24 hr). Thus, it would appear that enhanced PG formation is correlated with a reduced MCP-1 appearance. This functional relationship is unmasked by indomethacin causing the persistent enhancement of MCP-1 expression. The enhanced MCP-1 formation has functional and morphological consequences, that is, an increased production of glomerular chemotactic activity and a stimulated recruitment of M/M into the glomeruli. COX products in the glomerulus may therefore serve as endogenous repressors in the regulation of MCP-1 formation and may contribute to the healing process in this disease. Our data do not define which COX product may mediate MCP-1 expression; however,

Table 3. PGE_2 excretion in the urine

	PGE_2 *pg*	SD
Control	15756	6854
Nephritis	12591	10615
Nephritis + Mel	4892	1244
Nephritis + Indo	3086	965[ab]
Nephritis + SC	12546	4888

PGE_2 excretion in the urine of control rats, ATS nephritic rats, and ATS nephritic rats treated with indomethacin (Indo), meloxicam (Mel), or SC 58125 (SC) placed in metabolic cages overnight at day 5. Indomethacin significantly reduced PGE_2 excretion when compared to all other groups except nephritic rats treated with meloxicam. Results are expressed as pg PGE_2.

[a] $P < 0.02$ vs. nephritis

[b] $P < 0.005$ vs. nephritis + SC

Table 4. Number of monocytes/macrophages in glomeruli

	Time	ED1(+) cells	SD
Control		0.19	0.06
ATS	24 hours	3.98	0.63
ATS + Mel	24 hours	3.67	0.24
ATS + SC	24 hours	3.39	0.55
ATS + Indo	24 hours	4.90	0.44[a]
ATS	5 days	1.50	0.77
ATS + Mel	5 days	1.30	0.13
ATS + SC	5 days	0.81	0.17
ATS + Indo	5 days	3.01	0.80[b]
Control		0.19	0.04
Anti-GBM	5 days	0.83	0.08
Anti-GBM + Mel	5 days	1.22	0.30
Anti-GBM + Indo	5 days	1.88	0.87[c]

ED-1 positive cells per glomerulus (average of 50 glomeruli/kidney, 5 animals per group) in kidney sections from control, nephritic, and nephritic animals treated with different COX-inhibitors [indomethacin (Indo), meloxicam (Mel), SC 58125 (SC)]. M/M infiltration in glomeruli of ATS nephritic rats was significantly higher at 24 hours and 5 days compared with controls. Indomethacin significantly increased the appearance of M/M in glomeruli at both time points. The selective COX-2 inhibitors were without effect. In the anti-GBM nephritis indomethacin significantly increased the number of M/M in nephritic glomeruli at 5 days.

[a] $P = 0.04$ vs. nephritis

[b] $P = 0.02$ vs. nephritis + Mel, and vs. nephritis + SC

[c] $P = 0.02$ vs. nephritis

the fact that PGE_2 is the major product of rat glomeruli [34], together with the results of earlier studies demonstrating that PGs of the E series can suppress MCP-1 expression [14], suggests that PGEs are the primary candidates. The possibility that the changes induced by indomethacin are independent of COX inhibition still exists. We are not aware of data showing that MCP-1 mRNA expression is mediated by any COX-inhibitory drug independent of its effects on COXs.

It is, however, possible that COX inhibitors directly affect the regulation of transcription of chemokines. Aspirin has been shown to inhibit nuclear factor-κB (NF-κB) [35], one of the transcription factors that regulates MCP-1 expression [36]. We are not aware of data that show that indomethacin or the COX-2 inhibitors used in our experiments have effects on NF-κB. Nevertheless, this important issue of investigating how COX-inhibitors affect chemokine transcription will be addressed in separate experiments.

Because recent evidence from other models of inflammatory kidney diseases revealed that particularly products of COX-2 are important in inflammation [37, 38] and because indomethacin is an unselective COX-1/COX-2 inhibitor, we tried to further investigate whether one of the isoenzymes COX-1 or COX-2 may account for the repressive activity of MCP-1 formation. In contrast to the unselective COX-1 and COX-2 inhibitor indomethacin, the two selective COX-2 inhibitors used, Mel and SC, had less effects on chemokine expression. When higher doses of SC 58125 (6 mg/kg, compared with the 2 mg/kg body wt in other studies) were used, the difference between indomethacin and SC 58125 on glomerular MCP-1 mRNA expression diminished (Fig. 6). However, this dose-dependent effect of SC 58125 was less pronounced on chemotactic activity and mRNA expression of RANTES. We have no explanation for these differences, but it is possible that some effects are due to a possible unselective COX inhibition, because the compounds are not fully specific for COX-2 and also inhibit COX-1 at higher doses. Compared with indomethacin, the selective COX-2 inhibitors at the same concentration as indomethacin (2 mg/kg body wt) did not reduce urinary PGE_2 production significantly but were able to reduce PGE_2 production of isolated glomeruli *in vitro*, thereby showing biological activity. It seems possible that selective COX-2 inhibition does not contribute to the repressive effects on MCP-1 to the same degree or that COX-2 products do not use the same biosynthetic or signaling pathway as COX-1 products. There is intriguing evidence that, at least in some cell types, COX-2 is primarily localized in the nuclear membrane using a different source of substrate than COX-1, which is localized in a more diffuse pattern in the endoplasmatic reticulum and close to the cell membrane [39]. Inflammatory stimuli may thus activate COX-2, but COX-2 products may not be released on the surface of the cells where they can bind to prostaglandin receptors, and rather act directly in the nucleus to modulate cell differentiation. Studies in mesangial cell cultures demonstrating that the repressive effects of PGE_2 on MCP-1 mRNA expression are mediated by a prostacyclin E receptor–coupled stimulation of cyclic AMP [11] together with our results of less modified MCP-1 expression by treatment with COX-2 inhibitors might support our hypothesis that primarily COX-1 products regulate MCP-1 in this model of glomerulonephritis.

In summary, our data demonstrate that the inhibition of COX by indomethacin aggravates and maintains the expression of MCP-1 with consecutive enhancement of M/M infiltration in two rat models of glomerulonephritis. These data suggest that COX products are important repressors of stimulated MCP-1 in the diseased kidney. COX products may thus participate in the M/M clearing and in the healing process in glomerulonephritis.

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APPENDIX

Abbreviations used in this article are: ATS, antirat thymocyte serum; COX, cyclooxygenase; GBM, glomerular basement membrane; MCP-1, monocyte chemoattractant protein-1; Mel, meloxicam; M/M, monocyte/macrophage; PBS, phosphate-buffered saline; PGE, prostaglandin E.

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